Thermotaxis toward eccritic temperatures by the nematode *Caenorhabditis elegans*: a study in experiment replication

Stephen Bowlsby
Directed studies in biopsychology with C. Rankin,
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On a thermal gradient, *C. elegans* tends to migrate toward accustomed temperatures associated with food, and away from those associated with starvation. In order to test for the ability of *C. elegans* to learn associations between food and temperature, an attempt to replicate the original study that showed thermotaxic behavior was conducted under modified conditions. The following attempts at replication involved a series of experiments to identify confounds, an additional statistical analysis of nematode populations, and finally a literature review that resulted in a new hypothesis that a chemotactic factor in the modified transfer method caused the lack of migration in the majority of nematodes and the lack of predictability of the thermal preference of those that did migrate.

In their assay for mutant thermotaxis behavior in *C. elegans*, Hedgecock and Russell (1975) showed that thermotaxis can be modified by experience. On an agar-temperature gradient either hotter or colder than a previous growth temperature, worms accumulate preferentially on the edges proximal to that growth, or "eccritic", temperature. Conversely, populations that have passed through a swarming and dispersal stage in response to starvation at that same eccritic temperature move to the distal edges, away from the eccritic temperature.

Rankin and Chiba (1988) have shown several forms of non-associate learning by *C. elegans*, and a promising next step would be to show classical conditioning to a specified temperature, with food as the unconditioned stimulus. Rankin proposes (a) to test whether the worms learn to avoid an eccritic temperature associated with lack of food but not with starving, and then (b) to raise worms at a first temperature with food, a second temperature without food, and then see if on a gradient they choose the first temperature specifically. The first step in this process is to replicate the behavior described by Hedgecock and Russell (1975).

Materials and Methods

Hedgecock and Russell (1975) describe the 5-degree-per-10cm, aluminum-slab temperature gradient reproduced in this study. Worms were grown on agar plates at room temperature or on plates directly on the gradient for 5 hours with E. coli

food streaks aligned on a specific temperature. These were subsequently transferred onto non-food plates bracketting the recent, eccritic temperature. The 9-cm plates span approximately 5 degrees. Behavioral adaptation to a new eccritic temperature takes 5 hours, and may be associated with food or with no food.

Controls for population variations between plates included restriction to hermaphrodites and randomization by mixing colonies during the wash. All procedures of Hedgecock and Russell (the "original", 1975) were the same in this study (the "replication") except as noted under Modifications.

Scoring

As in the original study, each plate is treated as a single subject by representing it as a single "thermal preference" score on a scale of plus-100 to minus-100 (see Tables). The score represents the difference between the percentage of all migrating worms that arrived at the hot third of the plate and the percentage at the cold third. A positive thermal preference thus indicates a net movement toward the eccritic temperature if the plate is placed *below* that eccritic temperature on the gradient bar, while conversely the same positive thermal preference score indicates movement *away* from the eccritic temperature if the plate has been placed *above* that eccritic temperature on the gradient bar. Those worms that do not migrate from the center third are excluded from the calculation (see Tables).

Modifications

The worms were counted from a slowed-down panning videotape record made through a microscope, as opposed to the original method of counting from chloroformed plates . Both were done one hour after application to test plates.

Rather than using a small glass thermister to measure the equilibrated agar surface temperature, measurements were made using a small thermometer with its bulb imbedded in double-thickness test agar. This method indicated a consistent (+/- 0.5 degree) gradient generated by the apparatus, which varied negligibly relative to the uniform variation of 1 to 2 degrees over several hours produced by room temperature fluctuations. Fluctuations were controllable by constant monitoring of agar temperature.

Tests of the three eccritic temperatures (15, 20, and 25 degrees) cannot be done simultaneously on the slab; for time efficiency the 20 degree groups were taken from longterm room-temperature cultures and were tested prior to the slab-raised colonies (5 hours).

A less predictable constraint on this series of replication attempts was to perform the washing and transfer of the worms using only .5 % saline. This is similar enough, in this application, to the standard washing and transferring solution established by Brenner (1974), the "S medium", which is .585 % saline (0.1 M) along with other ions, antibiotics,

and buffer. However, the original experiments used, instead of S medium, nematode growth (NG) buffer, which has exactly the same inorganic ion content as the agar used in both studies. Hedgecock and Russell did not explain the rationale for this, nor for their use of Sephadex gel beads for the final transfer to the plate.

Results

A series of ten experiments

In the following descriptions of experiments, hypotheses refer to possible confounding factors in preceding experiments, and new controls refer to methodological changes adopted in an attempt to test those hypotheses to obtain results congruent with the original study.

Experiment 1: "F-20" (eccritic 20 degrees with food).
RESULT: Heat preference scores match Hedgecock and Russell's 3-hour starved groups (0 above eccritic, -50 below eccritic; c.f. Table 1); these scores do not match expected behavior of F-20 worms. Note that a day later the worms had all returned to the center saline-application site, and were swarming.

Experiment 2: F-20.
HYPOTHESES: 1. The equilibration time of the test plates in experiment #1 was shorter than in the original study (15 min. total with a 5 min. half-time, versus one hour in original), so that initial perception of the gradient was confused. 2. The heat preference of these colonies was reversed by a long starvation period known to have occurred prior to the final 24 hours with food. 3. The half-hour wash and transfer time may allow quick learning of an association of no food with 20 degrees, or instead an association with the raised room temperature near the experimental apparatus (which produces heat).
NEW CONTROLS: Use a 1-hour equilibration to match the original (hypothesis 1 above). Use (2) worms fed for 60 hours to extinquish any behavioral distruption caused by long starvation in the recent past. Use (3) a faster wash (10 min) in a second room with a more constant temperature where colonies are now kept, to reduce any quick associative learning due to wash conditions or to the raised temperature near the experimental apparatus.
RESULT: Heat preference scores match those of worms held without food in NG buffer for 6 hours in the original study (+50 above, -25 below; c.f. Table 1); still not the expected behavior.

Experiment 3: F-15,20,25. (Three eccritic temperatures.)
HYPOTHESES: 1. The batch of E. coli food streaks in colonies used in experiments #2 and #3 may have been inadequate; the worms were not fully fed or were even starving. 2. There may be an anomalous temperature-dependent

interaction effect specific to 20 degrees. 3. There may be an effect due to temporary mismatches between colony room temperature (3 degree range) and corresponding gradient temperatures as marked, which again would be specific to the 20-degree tests.

NEW CONTROLS: (1) Use new colonies with food-presence clearly distinguished and monitored. (2) Run the experiment at 15 and 25 degrees as well as at 20 degrees to control for any anomalous factor at 20 degrees. (3) Since the 15 and 25 degree plates are directly on the gradient slab for five hours their eccritic temperature is exactly defined, and corresponds exactly with the bracketting test plates after transfer, thus avoiding any inadvertant mismatches between eccritic and testing temperatures. Using plates eccritic at slab tempertures (15 and 25 degrees) also addresses hypothesis 1 since the eccritic temperature is known to have never been associated with lack of food.

RESULT: No evidence of behavior related to eccritic temperature (see Tables 1 & 2, and summary of results). No response from room-temperature-raised plates, or from the plates at the extreme ranges. One of the slab-raised plates showed a preference corresponding to expectation (but note that *both* plates, above and below, are needed to demonstrate eccritic response), and the tracks in its agar predominated on the same side. The only other responding plate, from a different eccritic temperature, showed a preference opposite to expectation -- but its tracks, in contrast, were on the expected side.

Experiment 4: F-20.

HYPOTHESES: 1. The more extreme temperatures in this experiment may affect worm behavior such that they cannot respond to the gradient below 15 degrees and above 25 degrees (this corresponds to reduced responses at these ranges shown in the original study). 2. The contradictory tracks observed in experiment #3 suggest that migration time toward the eccritic temperature may be much shorter in this replication, after which the worms then migrate differently. 3. Presence of food remaining in the saline upon application or after evaporation may disrupt resonse to the temperature gradient. 4. Duration in the saline wash has an unknown confounding effect.

NEW CONTROLS: (1) Concentrate on 20 degrees. (2) Monitor worm movements every 10 minutes to see if they are initially responding as expected but are then changing their migration patterns prior to the 1-hour assessment point. (3) Perform saline dilutions twice to remove any remaining food as well as try filtering the saline through filter paper and apply the paper directly to the agar to reduce transfer of food. (4) Duration in saline is minimized by the filter paper method. A problem here is that worms get lost inside the filter paper and produce a very small n, possibly traumatised, so this method was used only once, placed below a saline drop on one plate to compare. Additionally, with the large n obtained for this experiment it was observed that evaporation of the saline drop (about 2 minutes duration) brings many worms into close proximity and initiates swarming behavior,

which retards migration. Worms were therefore dispersed with a probe in such cases.

RESULTS: After 10 minutes on the plate above F-20 the worms that had not initially swarmed were at the hot edge (*away* from the eccritic temperature), fifteen of them up on the vertical slope; none were at the cold edge. By 20 minutes there was a turnover as the "pioneers" returned; still none at the cold, eccritic edge. By one hour these worms showed a random spread, where the central worms had moved very little (but are not damaged -- they respond normally to head-touch). On the plate below F-20 there was little migration by the vast majority of saline-deposited worms; only three out of 200 arrived at the hot and cold edges. Of the filter-deposited worms on the plate below F-20, about 40 % had migrated significantly toward the cold, non-eccritic side after half an hour, and not toward the other side; this showed more preference than the saline-deposited worms, but their average extent of migration was not clearly different, and none of them clearly migrated into the saline-deposit area.

Experiment 5: "NF-20" (No food at 20 deg.).

HYPOTHESES: 1. Worms may still have been partially starved in all preceding experiments, confusing their response. 2. Saline-wash duration and food in the saline deposit do not appear to be confounding variables, but another factor in the washing and transferring method may be important.

NEW CONTROLS: (1) Use 20-degree populations *starved* for 24 hours on unstreaked plates and test for expected *avoidance* of 20 degrees -- thus by this method their feeding condition is known for certain (and there is no food in the wash). (2) As a control for saline transferring, add a partial factorial design such that one equilibrated test plate is saline-transferred normally from a starved plate while the other starved plate is set *directly on the gradient slab to equilibrate*, with no other intervention.

RESULT: The plate below 20 degrees, which had *not* been transferred, showed a preference while the plate above, which *had* been transferred, did not. However, the preference that the non-transferred plate showed was *toward* the non-food eccritic temperature, thus still not congruent with the original study. (Table 3. See experiment 8 for completion of this transfer/non-transfer design across temperature ranges.)

Experiment 6: single F-20 worms.

HYPOTHESES: Worms in experiments #1-5 were not responding to the temperature gradient, but to unidentified factors, including a factor related to the saline transfer..

NEW CONTROLS: Observe single worms in different conditions, some with food on them, some with saline deposit, and some with neither. (These conditions were mixed with temperature conditions.)

RESULT: All worms remained isothermal for one hour. Worm pairs picked from food areas at 20 degrees and placed at extreme temperatures of 10 degrees and 30 degrees did not migrate toward eccritic temperature but did migrate isothermally up and down on the plate. Likewise for worm

pairs from NF-15 and from NF-25 which were picked out and placed at 20 degrees, and for pairs washed and transferred in saline from NF-15 and NF-25 to 20 degrees. These few individuals tested under different conditions did not migrate far enough along the gradient to detect the presence to the gradient.

Experiment 7: F-15,20,25.

HYPOTHESIS: Worms that had been *previously* starved at another time during their growth do not respond normally.

NEW CONTROLS: Repeat experiment #3 (food associated with three different temperatures) with worms from new colonies started from eggs only and maintained on an abundance of food on double streaked plates, so that any prior starvation is ruled out.

RESULT: No eccritic-temperature response was indicated. At F-15 and F-25 only the less extreme two plates showed a preference, as in experiment 3, with the only difference being a reversed preference in the plate below 25 degrees (see Table 2 and summary of results). (In this case it was clear from their tracks as well that on the hottest plate the worms traveled constantly and extensively all over the plate, whereas on the coldest plate they travelled very little, remaining centered.) One of the F-20 plates showed a slight avoidance this time (see Table 1 and summary of results).

Experiment 8: F-20.

HYPOTHESES: 1. Since the room-temperature colonies used for experiment #7 had just about exhausted their food supply at the time they were either transferred to fresh plates, or to gradient food plates for 5 hours at new eccritic temperatures (F-15&25), or directly to test plates for F-20, this near-exhaustion of food may have had an effect on the F-20 replication. 2. A larger n might be more revealing.

NEW CONTROLS: (1) Use worms from those re-plated to fresh plates as described above, and kept well-fed. (2) Obtain a large n from these plates.

RESULT: One plate showed a slight avoidance while the other showed no preference, the reverse of experiment 7, as summarized in Table 4; again not as expected.

Experiment 9: NF-20 complement to experiment 5.

HYPOTHESES: The test of no food at 20 degrees in experiment #5 with the transfer/no-transfer factor should be done again, reversing this latter independent variable to the converse temperature ranges (see experiment 5), so that temperature-range and transfer/no-transfer are controled independently. Experiments #5 and #9 should then be evaluated together as one experiment.

NEW CONTROLS: Repeat experiment 5 (NF-20) with transferring performed on the plate below and non-transferring on the plate above.

RESULT: As shown in Table 3, in the combined experiment comparison (#5 and #9), ***only the plates not subjected to saline transfer showed preferences.*** However, *both* plates of the pair had heat preferences (+), i.e., one moved toward the eccritic temperature, and one moved away from it -- the

preferences are still not congruent with the original. Note, however, that populations were not consistent; experiment 9 obtained a large n from starved plates that had gone into a "post-dispersal" state where huge, regularly spaced clumps of eggs in the center of the plate have hatched into about 100 densely swarming clumps of 15 or so larvae, while the adults have returned after dispersal to this site (Bowlsby, personal communication and video record).

Experiment 10: NF-20 post-swarming placed at 20.
HYPOTHESIS: An unidentified factor associated with washing and transfer in saline has a disruptive effect on the tendency of *C. elegans* to respond to temperature gradients. Since post-swarming (i.e., dispersing) colonies are highly motivated to disperse away from the starvation site even when there is no gradient, then post-swarming worms from 20 degrees, when saline-transferred to a 20-degree spot on a thermal gradient with no food, should quickly disperse from the point of application *unless* something is retarding them.
NEW CONTROLS: Apply saline-washed, post-swarming, NF-20 worms to a 20-degree plate *center* with 2.5 degrees of thermal gradient on either side across the plate.
RESULT: Of 60 worms, only *half* had dispersed outward to other temperatures after half an hour. Of the half that remained essentially isothermal, half of them remained in the spot delineated by the evaporated saline.

Statistics

A salient difference in results between the replication and the original was the percentage of worms on each plate that remained neutral, i.e., did not end up more than one degree away from the center starting point. In the original, less than 10 % of the worms remained near the point of application. In the replication, however, the mean percentage of non-migrating worms was 59, with a range of 20 - 86% (excluding swarms, see below), a standard deviation of 21 %, and a standard error of 6.3 %. Quite consistently, only about 40 % actually migrated. Therefore, because the thermal-preference score *excludes the majority of worms*, it was impossible to evaluate the meaning of such a score in this replication without employing an additional method of statistical analysis, as follows.

To quantitatively define which "subjects" (plates) had a preference and which did not, each plate had to first be treated as a population. Statistical significance was determined by assigning individual worms an integer score according to six vertical strips across the plate from cold to hot (see Tables). Thus in some cases a moderately high preference score was not statistically significant and is counted as no preference. A further confounding aspect to evaluation by statistics is that the additional statistical method used is based on a normal distribution, whereas the activity we are looking for should display a clearly *skewed* distribution. Thus in a few cases a population mean that differed significantly from the statistically random mean but

which did *not* show any skewed distribution across the gradient was rejected as indicating any thermal preference (see Tables).

The standard error of preference scores across repeated experiments by Hedgecock and Russell was 10 scale points. This compares to a standard error across the three F-20 experiments (#3, #7, and #8) of 21.5 points on plates below and 20.1 points on plates above.

TABLE 1.
Gradient preference after raising at 20 degrees with food.

Above 20 Deg:

Ex	Desc	n	Pr	1	2	3	4	5	6	X	s	S.E.	*t*	*p*	
1	stv?	105	0	15	15	20	15	30	10	3.57	1.60	.156	.450	>.50	
2	stv?	63	+57	2	4	7	28	17	5	4.09	1.12	.141	4.18	<.001	*
3	unstv	43	-20	3	2	29	8	0	1	3.12	.800	.122	3.11	<.01	R
7	NPS-1	32	+47	4	0	4	9	8	7	4.19	1.55	.274	2.52	<.02	*
8	NPS-2	279	-7	15	47	68	103	35	18	3.59	1.18	.070	1.28	.20	

Below 20 Deg:

Ex	Desc	n	Pr	1	2	3	4	5	6	X	s	S.E.	*t*	*p*	
1	stv?	95	-70	30	40	15	4	3	3	2.15	1.19	.122	11.0	~.001	*
2	stv?	80	-25	11	14	22	18	12	3	3.19	1.37	.153	.047	~1.00	
3	unstv	50	0	6	5	20	20	5	0	3.50	.810	.114	0	1.00	
7	NPS-1	58	+50	6	14	5	11	9	13	3.72	1.74	.228	.965	>.200	
8	NPS-2	114	-62	6	16	43	9	5	0	3.22	.930	.087	3.22	<.002	*

Column headings: experiment number (Ex); description (Desc); sample size (n); temperature preference, 100 x (H - C)/(H + C) (Pr); frequency in each of 6 vertical strips, cold to hot (1-6), where 1 & 2 are "C", 5 & 6 are "H", and 2 & 3 are neutral, not used in preference calculation; sample mean (X); standard deviation (s); standard error of the mean, $s/n^{1/2}$ (S.E.); t-score, absolute value of (X - 3.5)/ S.E. (*t*); and two-tailed probability of the null hypothesis, "mean = 3.5" (*p*). Statistical significance (based on normal distribution) is noted by "*". Significance rejected due to non-skewed distribution is noted by "R". Descriptions: "stv?" means this population may have been without food at time of transfer or starved, judging by response and by streaks on plates; "unstv" means unstarved, i.e., that food was available prior to transfer; "NPS" means this population is known to have been never previously starved.

TABLE 2.
Gradient preference after food at eccritic gradient temperatures 15 and 25 degrees (5 hours).

Eccritic 25 Degrees:

Ex	Desc	n	Pr	1	2	3	4	5	6	X	s	S.E.	*t*	*p*	
Above:															
3	unstv	85	0	8	11	30	20	7	9	3.40	1.37	.149	.670	~1.00	
7	NPS	41	+33	0	4	10	19	4	4	3.85	1.06	.166	2.11	.05	R
Below:															
3	unstv	207	-36	50	35	47	35	28	12	2.96	1.54	.107	5.04	<.001	*
7	NPS	35	+89	0	1	4	12	8	10	4.63	1.11	.188	6.01	<.001	*

(tracks contradict experiment 3)

Eccritic 15 Degrees:

Ex	Desc	n	Pr	1	2	3	4	5	6	X	s	S.E.	*t*	*p*	
Above:															
3	unstv	206	-87	34	75	50	40	7	0	2.57	1.08	.075	12.4	<.001	*
7	NPS	23	-100	5	6	7	5	0	0	2.52	1.08	.225	4.3	<.001	*
Below:															
3	unstv	284	-22	2	25	120	120	17	0	3.44	.766	.046	1.30	~.20	
7	NPS	90	+33	2	9	31	26	21	1	3.64	1.05	.111	1.26	>.20	

Column headings: experiment number (Ex); description (Desc); sample size (n); temperature preference, 100 x (H - C)/(H + C) (Pr); frequency in each of 6 vertical strips, cold to hot (1-6), where 1 & 2 are "C", 5 & 6 are "H", and 2 & 3 are neutral, not used in preference calculation; sample mean (X); standard deviation (s); standard error of the mean, $s/\sqrt{n}$ (S.E.); t-score, absolute value of (X - 3.5)/ S.E. (*t*); and two-tailed probability of the null hypothesis, "mean = 3.5" (*p*). Statistical significance (based on normal distribution) is noted by "*". Significance rejected due to non-skewed distribution is noted by "R". Descriptions: "unstv" means unstarved, i.e., that food was available prior to transfer; "NPS" means this population is known to have been never previously starved.

TABLE 3.

Gradient preference after 24 hours without food at 20 degrees.

Ex	Desc	n	Pr	1	2	3	4	5	6	X	s	S.E.	*t*	*p*	
Above:															
5	xfer	300	0	40	50	60	60	50	40	3.50	1.59	.092	0	1.00	
9	not	1880	+68	30	30	750	750	160	160	3.77	.990	.023	11.7	<.001	*
Below:															
9	xfer	1500	0	50	50	650	650	50	50	3.50			0	1.00	
5	not	277	+23	11	49	61	59	47	50	3.84	1.47	.088	3.86	<.001	*

Column headings: experiment number (Ex); description (Desc); sample size (n); temperature preference, 100 x (H - C)/(H + C) (Pr); frequency in each of 6 vertical strips, cold to hot (1-6), where 1 & 2 are "C", 5 & 6 are "H", and 2 & 3 are neutral, not used in preference calculation; sample mean (X); standard deviation (s); standard error of the mean, s/$\sqrt{n}$ (S.E.); t-score, absolute value of (X - 3.5)/ S.E. (*t*); and two-tailed probability of the null hypothesis, "mean = 3.5" (*p*). Statistical significance (based on normal distribution) is noted by "*". Significance rejected due to non-skewed distribution is noted by "R". Descriptions: "xfer" means saline-transferred; "not" means undisturbed population equilibrated on bar. Note: counts are estimated for first three plates, including larvae swarms.

TABLE 4.

Summary of the experiments using worms known to be unstarved at 20 degrees.

EXPERIMENT	BELOW 20 DEG	ABOVE 20 DEG
#3	no preference	no preference
#7	clear avoidance	no preference
#8	no preference	clear avoidance

Note: "Avoidance" is statistically significant with low *p* value. "No preference" is statistically significant with high *p* value (with the exception of #3 above-20, which was rejected). See Table 1 for explanation and *p* values.

Summary of results

As seen in Table 1. the only (two) significant never-previously-starved (NPS) F-20 plates had preferences *away* from the eccritic temperature. In none of the known-to-be-unstarved experiments was there a significant preference shown both above and below 20 degrees, as shown more graphically in Table 4.

Table 2 shows results of tests of eccritic temperatures above and below that of Table 1, all associated with food (F-15 and F-20). Only plates at the two more moderate temperature ranges showed significant preferences. This is similar to the reduced response found in the original study at these temperature ranges. The group above F-15 had a cold preference, but without a corresponding group below F-15 having a hot preference we cannot distinguish a cold preference from an eccritic-temperature preference. The only difference between nonstarved and NPS is on the plates below 25 degrees, in which the latter shows a hot preference as expected, while the former shows a cold preference. In this case, however, the tracks of the nonstarved plate contradict the worm's final distribution, matching instead the expected preference.

Table 3 shows that in situations not confounded by the issue of food, significant responses occurred only in those plates, at both temperatures, that had not been saline transferred. It should be noted, however, that even the plates not saline-transferred in this experiment had in the past originally been applied with saline in order to transfer the colony to a plate without food, and thus could still bear some of the distillate.

The *presence* of a thermal preference in these experiments cannot be seen to depend on presence or lack of food previously, nor on temperature range, except perhaps the extremes. Possible dependence of thermal response initiation on transfer method is shown only in the case of NF-20, whereas all other plates, whether they displayed a preference or not, were all saline transferred.

The *direction* of those preferences shown cannot be seen to be correlated with the method of transfer or with non-transfer; nor with associated prior food or lack of food; nor with a given temperature range, except perhaps with the plates above F-15.

No independent variable in this replication has been shown to have a main effect on the presence of migration or on the choice of direction if migration does occur. Finally, no correlation between plates bracketting a given temperature has been found. (i.,e., no behaviorally identified eccritic temperature).

Discussion

The original study reported mean preference scores of -75 on plates above and +75 on plates below eccritic temperatures associated with food. In comparison, the evidence of these

replication experiments is consistent with the hypothesis that, in these experiments, migration is determined by an unidentified variable or interaction of variables.

One possible factor in this apparent difference, however, is that the original results are means of repeated experiments with the same conditions. If plate preferences are inconsistent enough that they must be treated as individual subjects and predictable behavior is found only in the mean response of large samples, then the present replications may in fact be consistent with the original study. This can only be determined by a large number of repetitions of one experiment. It is perhaps telling that Hedgecock and Russell did not publish their repeated-plate sample size. Another possible difficulty may be in the general practice of excluding from the sample population those trials that responded in ways that were reasonably judged to be anomalies or in some way compromised. It would be useful to see the data from those trials selected *out* of the original study.

Assuming on the other hand that these replication attempts have been confounded by an unintentionally introduced variable or a failure to properly control the identified ones, the most probable locus is the saline transfer method. A further literature review initiated during the analysis of this data reveals a possible specific mechanism.

Hypothesis: a chemotaxis confound

The following new hypothesis may explain the existence of a powerful confounding effect produced in all replication experiments due entirely to the saline transfer method. *C. elegans is chemotactic toward NaCl* (Dusenbery et al, 1975). These worms readily choose between concentrations of 0.0 M and .01 M NaCl; the agar contains .05 M NaCl (3 gm/l) while the standard transferring S medium has 0.1 M NaCl, a difference of five times the difference that *C. elegans* can distinguish and move toward (Dusenbery et al, 1975). Thus the standard transferring method can be expected to have a confounding effect when other tactic or tropic responses are studied. Our .5% saline is .086 M NaCl, which is still .036 M higher than the agar, and would result in an even higher surface difference upon evaporation.

This chemotropic factor could explain (1) the observation that the majority of worms eventually return to within the boundary of the evaporated saline spot after the experiment; (2) the majority that fail to migrate far and the large number that in some plates do not move out of the salt-deposit boundary at all, particularly in experiment 10; (3) the results of table 3; and (4) rejection of significance based on a slightly deviating mean in non-skewed distributions -- this is likely the effect of an off-center saline deposit. It would explain why Hedgecock and Russell used NG buffer to wash and transfer (same inorganic ion content as the agar).

A second factor that the hypothesis of a NaCl chemotactic confound may explain is the unpredictable direction of preferences shown. ***Those worms that do migrate may be those with an impaired NaCl attraction.*** Hedgecock and Russell

(1975) showed that chemotactic-defective mutants had corresponding thermotactic abnormalities -- for example, the mutant DD73 is *repelled* by NaCl and also displays marked thermophilic mistaxis. The presence of greater surface NaCl in the center may therefore act as a selecting agent for a migrating subpopulation that is behaviorally abnormal.

In this light, the inconsistency of occurrence and of direction of thermal preference, as well as the lack of evidence for any one preferred temperature (as opposed to simply hotter or colder), may be due to at least three possible scenarios: (1) subpopulations that move toward preferred temperatures unrelated to their eccritic temperature; (2) subpopulations that migrate in consistent but unbounded temperature-gradient directions regardless of whether they are above or below their eccritic temperature; and (3) worms with no thermotactic response that migrate randomly away from the starting point (the "pioneers"), joined by others that simply "follow the leader".

These subpopulations may have arisen from founder effects during the continual restarting of colonies from one or a few individuals. Therefore this standard method of maintaining E. coli nutrient by re-plating worms from colonies that have depleted the nutrient may have the unfortunate side effect of occasionally starting a colony from an individual of unusual genotype.

Finally, a second chemotaxis confound may be at work: pH. *C. elegans* avoids acidity (Dusenbery, 1974). The standard S medium, agar, and NG buffer all contain potassium phosphate to maintain the pH at 6.0 (Dusenbery, 1975). Since the saline would be basic relative to the agar, perhaps another chemical attraction is involved in addition to the NaCl.

Suggested procedures

Three different methods may be used to control for NaCl chemotaxis. (1) Use a .3% saline transfer solution to match the agar NaCl concentration. Even though the surface concentration would increase upon evaporation, this concentration is the same as that used in the original experiments in the NG buffer. (2) Use distilled water to filter the worms quickly through a Millipore-type SC filter (pore size 8 microns) and transfer to the plate by touching the filter to the agar. This is the method employed by Dusenbery *et al.* (1975) during their chemotactic mutant assays; apparently *C. elegans* can easily handle short osmotic changes. (3) To control for effects of pH and other inorganic ions, NG buffer may be necessary.

Although swarming due to proximity caused during evaporation was not a confounding factor in many of the replication experiments, and although swarms that do occur can be separated by hand, it may be advisable to deposit the worms in a slurry of Sephadex beads, as in the original experiments, to insure separation.

Reference List

Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. Genetics. 77, 71-94.

Dusenbery, D.B., Sheridan, R.E., and Russell, R. L. 1975. Chemotaxis-defective mutants of the nematode *Caenorhabditis elegans. Genetics.* 80, 279-309.

Hedgecock, E.M., and Russell, R.L. 1975. Normal and mutant thermotaxis in the nematode *Caenorhabditis elegans. Proc. Nat. Acad. Sci. USA.* 72, 4061-4065.

Rankin, C.H., and Chiba, C.M. 1988. Short and long term learning in the nematode *C. elegans*. Abstract. Society for Neuroscience 1988 Annual Meeting. Toronto, Canada.

APPENDIX:
A Personal Learning Experience

Some wisdom is gained only by experience. This directed study project was my first attempt to perform a scientific experiment. I did not expect it to consist solely in attempting to replicate the results of previous investigators. Replication, I thought, would be like simply positioning the ladder so that I could climb the wall -- that would be done on the first day. I should have known better by virtue of my past career in film production, but the perspective I gained was all the more powerful by virtue of the immensity of my niavette.

Because I expected replication to be easy, variables were not completely controled. I started out winging it, always expecting the next partially-controlled test to give a nice clear response and I could get on to the real job. The process of being forced to add more and more controls, of becoming frustrated and bewildered, was a powerful experience in paradigm shifting. Clearly "biology is not wallpaper", as Cathrine Rankin had told me, but until I had directly experienced it myself, that dictum was an abstraction.

Likewise with time constraints. Because most of this one-term directed study had consisted of textbook learning, discussion, and participating in other already-working experimental procedures, the remaining time in which to perform and write up a new experiment with any guarantee of getting results was ridiculously small -- three weeks -- and so my frantic state at the time has taught me a warning about the time it takes to do these sorts of things. Now I know from direct experience why it takes so long to do a Ph.D.

This series of experiments also underlined the importance of continual and careful resource maintenance; of being familiar with the materials, such as what a good E. coli streak looks like; of keeping detailed records in order to refer back to details that did not seem of obvious significance at the time; in short, the craft of doing science.

I also gained an understanding of the importance of progressing in small, cautious steps, alert to the need to modify assumptions and goals. Methodical progression would have prevented the case where the two-factor experiment with temperature and transfer/no-transfer was done at different times (experiments 5 and 9) and thus by misfortune could not match the populations, introducing a third, uncontroled variable.

This project also gave me a first hand taste of how things can be discovered by experiments going wrong. I experienced "discovering" that C. elegans is chemotaxic toward NaCl. It came from a published paper, but it came right at the point that I had narrowed it down to that factor by trial and error; I had a taste of finally hitting on a cause after a lot of sweat.

Clearly, scientific publications do not always supply the reasons for their use of new methods. The lack of a stated

reason does not mean that the new method was simply a convenience and can be readily modified. Modifications of replications should be as carefully researched as would be methods for an original experiment. In a way this pointed out to me the importance of independent replication: there is no other way to test whether one investigator's controls and stated variables are really the only ones involved.

From the above consideration, another realization springs to mind that explains what I did not grasp before: the reason why negative results are not published. Prior to this I had the notion that if negative results were published it would save people from wasting time testing hypotheses that were false. But a negative result can be due to lack of realization of the exact conditions necessary, and publishing it can deter others from finding those conditions. If this had been the original test of the hypothesis that C. elegans is thermotaxic toward eccritic temperatures, it may have found no response. Thus Karl Popper's philosopy of science as falsification is double-edged: "failing to disprove" the scientific hypothesis is publishable; failing to disprove the null hypothesis is not. Because, after all, biology is not wallpaper.

Stephen Bowlsby
1107 - 2324 W. 1st Ave.,
Vancouver, B.C.
V6K 1G3 (604) 733-9441